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PCT

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<p>(54) Title: MODULATION OF VASCULAR CELL ADHESION MOLECULE EXPRESSION THROUGH NOVEL OLIGONUCLEOTIDE INTERACTIONS</p> <p>(57) Abstract</p> <p>Genes coding for vascular cell adhesion molecules, particularly VCAM-1, are modulated through interaction of oligonucleotides with transcriptional regulatory factors which bind to the genes. Specific and effective oligonucleotides are provided which interact with the transcriptional regulatory factors to diminish their interaction with the genes and downregulate their function. Multi-modal oligonucleotides are also provided which interact both with a transcriptional regulatory factor and with another aspect of gene function.</p>			

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5 **MODULATION OF VASCULAR CELL ADHESION MOLECULE EXPRESSION
THROUGH NOVEL OLIGONUCLEOTIDE INTERACTIONS**

FIELD OF THE INVENTION

The present invention is directed to the field of
10 oligonucleotide therapeutic compositions and methods.
Modulation, especially down-regulation, of genes or gene
portions associated with disease states is a particular object
of the invention. The invention provides therapeutic and
research compositions which interact with certain
15 transcriptional regulatory factors to modulate transcription of
vascular cell adhesion molecule genes. Chimeric compositions
having multi-modalities are also provided which can affect a
plurality of gene functions by interfering with splicing or
translation as well as transcription. Treatment of inflammatory
20 and vascular diseases is a preferred embodiment of the present
invention.

BACKGROUND OF THE INVENTION

Certain cellular adhesion molecules have been
25 implicated in inflammatory and vascular diseases. As an
example, a major mediator of the atherosclerotic process is the
expression of specific adhesion molecule proteins on the surface
of vascular endothelial cells. These adhesion molecules bind to
immune cells, termed leukocytes, and initiate and propagate the
30 inflammatory response that is likely central to both
atherosclerosis and post-angioplasty restenosis. One of these
vascular cell adhesion molecules, VCAM-1, plays an especially
important role in atherosclerosis by binding a specific class of
leukocytes, termed mononuclear leukocytes. Multiple signals
35 induce the expression of VCAM-1. ICAM-1 and E-selectin are

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other inducible vascular cell adhesion molecules that mediate other aspects of general inflammatory response.

It is greatly desired to achieve palliative, diagnostic and therapeutic regimes for inflammatory disorders, 5 atherosclerosis, restenosis and other diseases through modulation of the production of vascular cell adhesion molecules by cells. Specificity of such therapy is also greatly desired.

Oligonucleotides have recently become accepted as therapeutic moieties in the treatment of disease states in 10 animals and man. For example, workers in the field have now identified antisense, triplex and other oligonucleotide therapeutic compositions which are capable of modulating expression of genes implicated in viral, fungal and metabolic diseases. For example, PCT patent applications PCT/US90/ 07067 15 filed December 3, 1990; US91/01327 filed February 25, 1991; and PCT/US91/05815 filed August 14, 1991 disclose therapeutic oligonucleotides for treatment of papillomavirus, herpesvirus and cytomegalovirus infections respectively.

PCT patent application PCT/US91/05802 Filed August 15, 20 1991 discloses oligonucleotides for treatment of Candida infections. PCT patent application US91/02628 filed April 17, 1991 and PCT/US91/05209 filed July 23, 1991 are directed to modulation of genes regulating lipid metabolism and cell adhesion respectively employing oligonucleotides as therapeutic 25 agents. United States Serial No. 007,997 filed January 21, 1993 , United States Serial No. 063,167 filed May 17, 1993 and PCT patent application US93/08101 are also directed to oligonucleotide therapeutics relating to cellular adhesion molecules.

30 U.S. Patent 5,098,890, in the name of Gewirtz, et al., is directed to antisense oligonucleotide therapies for certain cancerous conditions, while U.S. Patent 5,166,195 issued November 24, 1992 provides oligonucleotide inhibitors of HIV. It is, thus, established that oligonucleotides can be useful 35 therapeutic instrumentalities and that the same can be configured to be useful in treatment regimes for treatment of cells and animals, especially humans.

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It is apparent that oligonucleotide therapeutics is an accepted modality for treatment of disease. Methods of drug delivery and of modification to permit therapeutically significant residencies are now known. Notwithstanding the acceptance of oligonucleotide therapeutics, additional, effective approaches to gene modulation, especially down-regulation, employing oligonucleotides are greatly desired. Moreover, mechanisms of action for oligonucleotide therapeutics different from classical antisense and triplex mechanisms are also greatly desired.

Transcriptional regulatory factors (transcription factors) are DNA binding proteins which transduce extracellular signals into nuclear regulatory signals. Normally, transcriptional regulatory factors are activated or synthesized, translocate to the nucleus of cells, and bind to specific DNA sequences called enhancer or regulatory elements. Once bound, the transcriptional regulatory factor modulates gene transcription.

Wu, et al. "Inhibition of in Vitro Transcription by Specific Double-Stranded Oligodeoxy-ribonucleotides," Gene 89 203-209 (1990) used double stranded deoxyoligonucleotides to compete for binding of nuclear factors to specific promoter and enhancer elements. The authors tested the effect of oligonucleotide length, sequence and number of nuclear factor binding sites on in vitro transcription of adenovirus; potential therapeutic regimes were discussed. See also Holcemberg and Wu, WO 91/11535 which also discloses phosphorothioate derivatives in this context. Chu et al. "Binding of Hairpin and Dumbbell DNA to Transcription Factors" Nucleic Acids Research 19 6958 (1991) discloses the use of single stranded DNA in hairpin loop and ligated circular (dumbbell) forms for binding to transcription factors. See also Chu and Orgel, WO 92/18522.

Others have approached therapeutics through oligonucleotide interaction with transcription regulatory factors. Taniguchi T. U.S. Patent 5,157,115 issued 10/20/92 discloses nucleic acid compositions which inhibit or control the

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IL-2 or IL-2a genes by competitively binding to their respective transcription factors.

Blumenfeld et al. in WO 92/19732 and WO 91/15114 disclose antisense or sense oligonucleotides which can have a 5 protein binding sequence.

Androphy et al. in European Patent Application 302,758 disclose nucleic acids or proteins for inhibiting the growth of a virus, such as papillomavirus, by preventing binding of a stimulatory protein encoded by the DNA of the virus (which protein would, if bound, enhance viral transcription) to the DNA 10 of the virus. See also Androphy, et al., Nature 325:70-73 (1987) and Androphy et al., WO 91/14790, relating to inhibitors of transcription activation activity of papilloma viruses. Clusel, et al., Nucleic Acids Research 21:3405-3411 (1993) disclose ex 15 vivo regulation of specific gene expression by nanomolar concentration of double-stranded dumbbell oligonucleotides containing a hepatic nuclear factor-1 binding site.

Bielinska, et al., Science 250:997-1000 (1990) regulated gene expression with double-stranded phosphorothioate 20 oligonucleotides. Inhibition of sequence-specific DNA-binding proteins with oligonucleotides containing octamer or kB consensus sequences, which bound specifically either octamer transcription factor or NF-kB, were disclosed to inhibit HIV enhancer activation or IL-2 secretion.

Eck et al., Molecular and Cellular Biology 13:6530- 25 6536 (1993), used high doses ($\geq 20 \mu\text{M}$) of a double-stranded 38mer phosphorothioate oligonucleotide containing three tandem repeats of the NF-kB consensus binding sequence to inhibit the expression of both ICAM-1 and CD11b, a leukocyte integrin, in 30 human endothelial cells in culture.

While interaction with transcriptional regulatory factors by DNA oligonucleotides has been known heretofore, and while therapies have been predicted to be possible, there have been no specific oligonucleotides and no effective methods using 35 oligonucleotides for interaction with transcriptional regulatory factors which can effectively and specifically target vascular cell adhesion molecules, particularly VCAM-1, and diseases for

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which such molecules are implicated. Such materials and methods are greatly desired.

It is an object of the invention to provide oligonucleotide diagnostic and therapeutic compositions and 5 methods for modulating expression of genes associated with disease states implicating vascular cell adhesion molecules, particularly VCAM-1.

A further object is to provide such oligonucleotides which interact with one or more transcription factors to effect 10 such modulation.

Compositions and methods for treatment of inflammatory and vascular diseases as well as other disease states for which vascular cell adhesion molecules are implicated are also objects of the invention.

15 Yet another object is to afford oligonucleotide therapeutic compositions and treatment regimes which are directed to a plurality of gene portions in a multi-modal therapeutic approach.

Other objects will become apparent from review of the 20 instant specification.

SUMMARY OF THE INVENTION

This invention relates to compositions and methods for modulating gene expression relating to vascular cell adhesion 25 molecules. The expression of selected genes is modulated through interaction of novel oligonucleotides with a transcriptional regulatory factor appertaining to the gene. The invention is also directed to multimodal oligonucleotides and for methods for their employment for the modulation of 30 preselected genes. Such multimodal oligonucleotides are capable of operating to downregulate or otherwise modulate such genes at a plurality of levels, such as splicing of pre-mRNA or translation of mRNA, preferably including interaction with at least one transcriptional regulatory factor.

The present invention also provides methods of diagnosis and therapy employing such oligonucleotides such that 35 diseases or body states for which expression or overexpression

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of a gene coding for a vascular cell adhesion molecule are implicated can be diagnosed, palliated or cured through administration of oligonucleotides of the invention to cells or mammals, preferably humans.

5 In accordance with embodiments of this invention, methods for modulating expression of a gene coding for a vascular cell adhesion molecule are provided, which comprise selecting a portion of the gene. The gene is one which has a DNA sequence which binds a member of a family of transcriptional
10 regulatory factors. Cells containing the gene are then contacted with an oligonucleotide moiety substantially identical in sequence to at least a portion of said binding sequence of the gene. The oligonucleotide moiety preferably comprises either two complementary strands of DNA or a single strand of DNA which
15 is self-complementary.

In order that the oligonucleotide not be rapidly degraded by nucleases in the cells, it is preferred that the oligonucleotides be modified. Thus, modification of at least some of the inter-nucleotide linking groups, which are normally phosphodiesters is preferred. Phosphorothioate modifications, which are known *per se* to persons of skill in the nucleic acid art, are preferred. Other modifications to the linkages, and other modifications to the oligonucleotides, including sugar or base modifications, known to persons of skill in the art may
25 also be employed.

While not wishing to be bound by theory, it is believed that modulation of expression of the gene in accordance with the present invention is effected through inhibition of transcription of the gene. This is believed to take place
30 through interference with the normal effect of the transcriptional regulatory factor upon the gene.

It has been found to be possible to modulate genes which code for vascular cell adhesion molecules, particularly VCAM-1, ICAM-1 and E-selectin. In some embodiments, the
35 transcriptional regulatory factor preferably is a member of the nuclear factor kB (NF-kB) family of transcriptional regulatory factors. Interference with other factors which also bind

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specific DNA sequences in the promotor region of vascular cell adhesion molecule genes is also believed to be effective in the practice of one or more aspects of the present invention. In preferred embodiments, these other factors include GATA, AP-1, 5 Sp-1, AP-2, AP-3, interferon response factors, octamer transcription factor and NF-ELAM1. Specific DNA sequences include the ets binding sequence, TATA box, inverted CCAAT sequence, AP-1 binding site, NF-ELAM1 binding site, KR, KL, a CAT sequence, an Sp-1 binding site, AP-2 binding site, AP-3 10 binding site, interferon response element, octamer binding sequence and the GATA binding site.

In accordance with other aspects of the invention, oligonucleotide moieties are provided comprising a nucleotide sequence which is substantially identical to the sequence of at 15 least a portion of a transcriptional regulatory factor binding sequence of a gene encoding a vascular cell adhesion molecule, particularly VCAM-1, ICAM-1 or E-selectin.

Multimodal oligonucleotides for modulation of gene expression are also contemplated by the present invention. 20 Oligonucleotide moieties in accordance with these embodiments comprise a plurality of regions; i.e., they are chimeric. A first region has a sequence which is substantially identical to at least a portion of the sequence of a gene, which gene sequence binds a transcriptional regulatory factor. A second 25 region of the oligonucleotide moiety is constructed to be specifically hybridizable either with mRNA deriving from the gene, or with a splicing region of pre-mRNA deriving from the gene. It is preferred that the first and second regions be capable of being liberated, one from the other, preferably in a 30 cellular process, such that upon entry into cells, the chimeric oligonucleotides separate into several independent, effective subunits representing the first and second regions. This may be done by causing the first and second regions to flank a junction region in the oligonucleotide, which junction region is a 35 substrate for a cellular metabolic process. For example, if the junction region is a substrate for RNase H, upon entry into cells cleavage of the multimodal, chimeric oligonucleotides will

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liberate the first and second regions. To this end, it is preferred to stabilize the first and second regions against nuclease degradation and other forms of destruction such as by use of phosphorothioates, methyl phosphonates, peptide-nucleic acid (PNA) or other modified forms of nucleotides. "Hybridization," in the context of this invention, means hydrogen bonding, also known as Watson-Crick base pairing, between complementary bases, usually on opposite nucleic acid strands or two regions of a nucleic acid strand. Guanine and cytosine are examples of complementary bases which are known to form three hydrogen bonds between them. Adenine and thymine are examples of complementary bases which are known to form two hydrogen bonds between them. "Specifically hybridizable" indicates a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences. It is understood that an oligonucleotide need not be 100% complementary to its target nucleic acid sequence to be specifically hybridizable.

In accordance with other preferred embodiments, the chimeric oligonucleotides of the invention include at least a first and a second region, each having a DNA sequence substantially identical to a gene promotor region sequence which binds a different transcriptional regulatory factor, e.g. the regulatory factor which binds to the first region is different from the factor which binds to the second region. The first and second regions may be linked by stabilized internucleotide linkages such as phosphorothioate linkages. In other embodiments, the first and second regions are linked by a junction region which is a substrate for a cellular metabolic process, resulting in liberation of the first and second regions upon entry into cells.

In accordance with still other preferred embodiments, the chimeric oligonucleotides of the invention include three or more regions, at least one of which preferably has its sequence substantially identical to at least a portion of a transcriptional regulatory binding sequence on the gene to be modulated. The second, third, and other regions of the

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oligonucleotide may be the same or different and may comprise additional transcription factor binding sites or may be directed to interaction with splicing sites on pre-mRNA or to mRNA itself. It will be appreciated that the interaction of such 5 oligonucleotide regions with pre-mRNA or mRNA is likely to occur in an antisense fashion, i.e., through specific hybridization with the mRNA or pre-mRNA.

Thus, the chimeric oligonucleotides of the invention may, in accordance with preferred embodiments, operate through 10 distinctly different mechanisms. One mechanism can be an antisense mechanism directed towards pre-mRNA or mRNA while the other mechanism can be seen to scavenge transcriptional regulatory factor to prohibit such factor from interacting with genes to cause transcription thereof. The overall effect is to 15 modulate expression of selected genes in a multi-modal fashion, that is, through operation of a plurality of different mechanisms. In accordance with other embodiments of the invention, methods for treating mammals, especially humans, which are suspected of having a disease involving expression of 20 a selected gene are provided. Compositions including oligonucleotide moieties in accordance with the invention are administered to the mammal, usually in a pharmaceutically acceptable carrier or diluent and in accordance with therapeutic regimes, which effect palliation or cure of the disease. In 25 accordance with one embodiment, inflammatory diseases can be treated such as atherosclerosis, restenosis and other inflammatory diseases in mammals, especially humans. In such case, one effective transcriptional regulatory factor for which interaction is preferred is a member of the NF- κ B family of 30 regulatory factors.

Inflammation is a localized protective response elicited by tissues in response to injury, infection, or tissue destruction resulting in the destruction of the infectious or injurious agent and isolation of the injured tissue. A typical 35 inflammatory response proceeds as follows: recognition of an antigen as foreign or recognition of tissue damage, synthesis and release of soluble inflammatory mediators, recruitment of

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inflammatory cells to the site of infection or tissue damage, destruction and removal of the invading organism or damaged tissue, and deactivation of the system once the invading organism or damage has been resolved. In many human diseases 5 with an inflammatory component, the normal, homeostatic mechanisms which attenuate the inflammatory responses are defective, resulting in damage and destruction of normal tissue.

Cell-cell interactions are involved in the activation 10 of the immune response at each of the stages described above. One of the earliest detectable events in a normal inflammatory response is adhesion of leukocytes to the vascular endothelium, followed by migration of leukocytes out of the vasculature to the site of infection or injury. The adhesion of these 15 leukocytes, or white blood cells, to vascular endothelium is an obligate step in the migration out of the vasculature. Harlan, J.M., *Blood* 1985, 65, 513-525. In general, the first inflammatory cells to appear at the site of inflammation are neutrophils followed by mononuclear leukocytes, and lymphocytes. 20 Cell-cell interactions are also critical for propagation of both B-lymphocytes and T-lymphocytes resulting in enhanced humoral and cellular immune responses, respectively.

The adhesion of white blood cells to vascular 25 endothelium and other cell types is mediated by interactions between specific proteins, termed "adhesion molecules," located on the plasma membrane of both white blood cells and vascular endothelium. These vascular cell adhesion molecules bind to immune cells, termed leukocytes, and initiate and propagate the inflammatory response that is likely central to both 30 atherosclerosis and post-angioplasty restenosis.

Vascular cell adhesion molecules are involved in the adherence of white blood cells to vascular endothelium and subsequent migration out of the vasculature. Vascular cell adhesion molecules identified to date include intercellular 35 adhesion molecule 1 (ICAM-1), intercellular adhesion molecule 2 (ICAM-2), intercellular adhesion molecule 3 (ICAM-3), E-selectin [previously known as endothelial leukocyte adhesion molecule-1

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(ELAM-1)], vascular cell adhesion molecule-1 (VCAM-1), L-selectin (MEL-14) and P-selectin (granule membrane protein-140, GMP-140) and their respective receptors. The adherence of white blood cells to vascular endothelium appears to be mediated in part if not in toto by ICAM-1, ICAM-2, E-selectin, VCAM-1 and P-selectin. Dustin and Springer, *J. Cell Biol.* 1987, 107, 321-331.

P-selectin and E-selectin are primarily involved in the adhesion of neutrophils to vascular endothelial cells. VCAM-1 primarily binds T and B lymphocytes. VCAM-1 plays an especially important role in atherosclerosis by binding a specific class of leukocytes, termed mononuclear leukocytes. In addition, VCAM-1 may play a role in the metastasis of melanoma, and possibly other cancers. ICAM-1 plays a role in adhesion of neutrophils to vascular endothelium, as well as adhesion of mononuclear leukocytes and lymphocytes to vascular endothelium, tissue fibroblasts and epidermal keratinocytes. ICAM-1 also plays a role in T-cell recognition of antigen presenting cell, lysis of target cells by natural killer cells, lymphocyte activation and proliferation, and maturation of T cells in the thymus. In addition, recent data have demonstrated that ICAM-1 is the cellular receptor for the major serotype of rhinovirus, which account for greater than 50% of common colds. Staunton et al., *Cell* 1989, 56, 849-853; Greve et al., *Cell* 1989, 56, 839-847.

Expression of ICAM-1, E-selectin and VCAM-1 has been associated with a variety of inflammatory skin disorders such as allergic contact dermatitis, fixed drug eruption, lichen planus, and psoriasis; Ho et al., *J. Am. Acad. Dermatol.* 1990, 22, 64-68; Griffiths and Nickoloff, *Am. J. Pathology* 1989, 135, 1045-1053; Lisby et al., *Br. J. Dermatol.* 1989, 120, 479-484; Shiohara et al., *Arch. Dermatol.* 1989, 125, 1371-1376. In addition, ICAM-1, E-selectin and VCAM-1 expression has been detected in the synovium of patients with rheumatoid arthritis; Hale et al., *Arth. Rheum.* 1989, 32, 22-30; ICAM-1 expression has been detected in pancreatic B-cells in diabetes; Campbell et al., *Proc. Natl. Acad. Sci. U.S.A.* 1989, 86, 4282-4286; thyroid

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follicular cells in patients with Graves' disease; Weetman et al., *J. Endocrinol.* 1989, 122, 185-191; and expression of ICAM-1 and VCAM-1 has been associated with cardiac, renal and liver allograft rejection and in early atherosclerotic lesions.; Faull 5 and Russ, *Transplantation* 1989, 48, 226-230; Adams et al., *Lancet* 1989, 1122-1125; O'Brien et al., *J. Clin. Invest.* 1993, 92, 945-951; Libby et al., *J. Clin. Invest.* 1993, 92, 538-539.

It has been hoped that inhibitors of ICAM-1, VCAM-1 and E-selectin expression would provide a novel therapeutic 10 class of anti-inflammatory agents with activity towards a variety of inflammatory diseases or diseases with an inflammatory component such as asthma, rheumatoid arthritis, atherosclerosis and restenosis, allograft rejections, various dermatological conditions and psoriasis. In addition, 15 inhibitors of ICAM-1, VCAM-1, and E-selectin may also be effective in the treatment of colds due to rhinovirus infection, AIDS, and some cancers and their metastasis. To date, there are no known therapeutic agents which effectively prevent the expression of the cellular adhesion molecules E-selectin, VCAM-1 20 and ICAM-1. The use of neutralizing monoclonal antibodies against adhesion molecules in animal models provide evidence that such inhibitors, if identified, would have therapeutic benefit for asthma; Wegner et al., *Science* 1990, 247, 456-459 and renal and cardiac allografts; Cosimi et al., *J. Immunol.* 25 1990, 144, 4604-4612. The use of a soluble form of ICAM-1 molecule was also effective in preventing rhinovirus infection of cells in culture. Marlin et al., *Nature* 1990, 344, 70-72.

Current agents which affect intercellular adhesion 30 molecules include synthetic peptides, monoclonal antibodies, and soluble forms of the adhesion molecules. Monoclonal antibodies may prove to be useful for the treatment of acute inflammatory response due to expression of ICAM-1, VCAM-1 and E-selectin. However, with chronic treatment, the host animal develops 35 antibodies against the monoclonal antibodies thereby limiting their usefulness. In addition, monoclonal antibodies are large proteins which may have difficulty in gaining access to the inflammatory site. Soluble forms of the cell adhesion molecules

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suffer from many of the same limitations as monoclonal antibodies in addition to the expense of their production. Thus, there is a long felt need for molecules which effectively inhibit vascular cell adhesion molecules. In particular, there
5 is a long felt need for molecules which specifically inhibit VCAM-1, E-selectin and ICAM-1 expression. Therapeutic oligonucleotides can avoid many of the pitfalls of current agents used to block the effects of ICAM-1, VCAM-1 and E-selectin. This need has been partly satisfied through the use
10 of antisense oligonucleotide interactions with mRNA coding for cellular adhesion molecules as reflected in patent applications owned by the assignee of this invention, referred to *supra* and incorporated herein by reference. There remains, however, a great need for further oligonucleotide therapeutics for cellular
15 adhesion molecule-related body states and diseases.

Human ICAM-1 is encoded by a 3.3-kb mRNA resulting in the synthesis of a 55,219 Dalton protein the entirety of which is hereby incorporated by reference. ICAM-1 is heavily glycosylated through N-linked glycosylation sites. Staunton et
20 al., *Cell* 1988, 52, 925-933. ICAM-1 is a member of the immunoglobulin supergene family, containing 5 immunoglobulin-like domains at the amino terminus, followed by a transmembrane domain and a cytoplasmic domain. The primary binding site for LFA-1 and rhinovirus are found in the first immunoglobulin-like
25 domain. However, the binding sites appear to be distinct. Staunton et al., *Cell* 1990, 61, 243-354. ICAM-1 exhibits a broad tissue and cell distribution, and may be found on white blood cells, endothelial cells, fibroblast, keratinocytes and other epithelial cells. The expression of ICAM-1 can be
30 regulated on vascular endothelial cells, fibroblasts, keratinocytes, astrocytes and several cell lines by treatment with bacterial lipopolysaccharide and cytokines such as interleukin-1, tumor necrosis factor, gamma-interferon, and lymphotoxin. See, e.g., Frohman et al., *J. Neuroimmunol.* 1989,
35 23, 117-124. The molecular mechanism for increased expression of ICAM-1 following cytokine treatment has not been well characterized.

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E-selectin is a 115-Kda membrane glycoprotein which is a member of the selectin family of membrane glycoproteins. Bevilacqua et al., *Science* 1989, 243, 1160-1165 the entirety of which is hereby incorporated by reference. The amino terminal 5 region of E-selectin contains sequences with homologies to members of lectin-like proteins, followed by a domain similar to epidermal growth factor, followed by six tandem 60-amino acid repeats similar to those found in complement receptors 1 and 2. These features are also shared by P-selectin and L-selectin 10 antigen, a lymphocyte homing antigen. E-selectin is encoded for by a 3.9-kb mRNA. The 3'-untranslated region of E-selectin mRNA contains several sequence motifs ATTTA which are responsible for the rapid turnover of cellular mRNA consistent with the transient nature of E-selectin expression.

15 E-selectin exhibits a limited cellular distribution in that it has only been identified on vascular endothelial cells. Like ICAM-1, E-selectin is inducible by a number of cytokines including tumor necrosis factor, interleukin-1 and lymphotoxin and bacterial lipopolysaccharide. In contrast to 20 ICAM-1, E-selectin is not induced by gamma-interferon. Bevilacqua et al., *Proc. Natl. Acad. Sci. USA* 1987, 84, 9238-9242; Wellicome et al., *J. Immunol.* 1990, 144, 2558-2565. The kinetics of E-selectin mRNA induction and disappearance in human umbilical vein endothelial cells precedes the appearance and 25 disappearance of E-selectin on the cell surface.

VCAM-1 is a 110-Kda membrane glycoprotein encoded by a 3.2-kb mRNA (Figure 3). VCAM-1 appears to be encoded by a single-copy gene. Osborn et al., *Cell* 1989, 59, 1203-1211 the entirety of which is hereby incorporated by reference. Like 30 ICAM-1, VCAM-1 is a member of the immunoglobulin supergene family, containing seven immunoglobulin-like domains of the H type. The receptor for VCAM-1 is proposed to be VLA4 integrin as demonstrated by the ability of monoclonal antibodies to VLA4 to block adherence of Ramos cells to VCAM-1. VCAM-1 is 35 expressed on activated vascular endothelial cells and nonendothelial cells. Like ICAM-1 and E-selectin, expression of VCAM-1 on vascular endothelium is regulated by treatment with

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cytokines. Rice and Bevilacqua, *Science* 1989, 246, 1303-1306; Rice et al., *J. Exp. Med.* 1990, 171, 1369-1374. Increased expression appears to be due to induction of the mRNA.

Transcriptional regulatory factors, also known as transcription factors, the proteins which transduce extracellular signals into nuclear regulatory signals, are known *per se* and have been the subject of several reviews. For example, see Pabo, C.O. and Sauer, R.T., *Transcription factors: Structural Families and Principles of DNA Recognition* Annu. Rev. Biochem. 61:1053-1095 (1992); Morimoto, R.I., *Transcription Factors: Positive and Negative Regulators of Cell Growth and Disease*, Current Opinion in Cell Biology 4:480-487 (1992); and Hayashi, T., Ueno, Y., and Okamoto, T. *Oxidoreductive Regulation of Nuclear Factor kB*, J. Biol. Chem. 268:11380-11388 (1993), all of which are incorporated herein by reference to provide disclosure as to transcriptional regulatory factors, the nuclear factor kB (NF-kB) family and the interactions of the foregoing with nucleic acids.

NF-kB is an example of a family of eukaryotic transcriptional regulatory factors. NF-kB was discovered by Sen and Baltimore, *Cell* 1986, 46, 705-716, and was found to be a B cell-specific DNA binding protein that recognized a cis-regulatory element within the enhancer of the immunoglobulin gene. Subsequently, it has been shown that NF-kB is not restricted to B cells, but is inducible in a wide variety of cells. Latent NF-kB is a trimeric protein consisting of IkB, a cytosolic inhibitor of kB, and at least two distinct DNA binding proteins. Latent NF-kB becomes activated upon loss of IkB binding to dimeric NF-kB. IkB is an inhibitor of nuclear translocation for the DNA binding dimer. To date, at least three forms of DNA binding proteins have been identified which can form both homodimers and heterodimers, p50, p65 and rel. It is likely that other closely related gene products will be identified as well. Thus, NF-kB and proteins having similar structure or effect may be described as a family of proteins. Other examples of transcriptional regulatory factors are GATA, AP-1, Sp-1, AP-2, AP-3, interferon, octamer transcription factor

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and NF-ELAM1.

The promotor regions of the ICAM-1, E-selectin and VCAM-1 genes have been described, and all contain numerous regulatory elements. Of these, some have been well defined and
5 others are putative transcription factor binding sites or consensus binding sequences.

Regulatory elements present in the 5' region of the human ICAM-1 gene include two TATA boxes located at bases -70 and -352 (i.e., 70 bases and 352 bases upstream or towards the
10 5' end of the translation initiation site) and a CAT-like sequence at -397. Computer analysis revealed two Sp-1 binding sites located at positions -246 and -99 and two sequences with homology to consensus AP-1/TRE binding sites at positions -1294 and -324. Voraberger et al., (1991) J. Immunol. 147:2777-2786;
15 Degitz et al., (1991) J. Biol. Chem. 266:14024-14030. There are also sequences homologous to AP-2 binding sites at position -88 and an AP-3 like consensus binding site at position -414. A potential NF- κ B binding site is found at position -540 and a potential interferon response element (IRE) is at position -109.
20 The cis regulatory elements that are responsible, in part, for the cytokine responsiveness of the ICAM-1 gene are between position -300 and -1205. Cornelius et al., (1993) J. Invest. Dermatol. 100:753-758. Identified transcriptional regulatory factors that bind sequences in this region include NF- κ B and AP-
25 1. It is possible that additional DNA binding proteins interact with sequences in this region.

The E-selectin gene contains several consensus sequences for known DNA binding proteins. Located 97 bases upstream of the transcription initiation site is an inverted
30 CCAAT consensus sequence (ATTGG; this promoter element is known to function in either orientation). A putative TATA box lies 30 bases upstream of the transcription initiation site. Collins et al., J. Biol. Chem. 1991, 266, 2466-2473. A putative NF- κ B binding element is present at position -94 and a putative AP-1
35 binding site is present at position -495. A DNA binding factor (NF-ELAM1) which recognizes DNA sequence in common with a T-cell enhancer is found at position -150 and in conjunction with the

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E-selectin NF- κ B sequence was found to be sufficient to confer cytokine inducibility to a reporter gene construct transfected into endothelial cells. Hooft van Huijsduijnen et al., J. Biol. Chem., 1992, 267, 22385-22391.

5 A number of regulatory elements have been described on the VCAM-1 gene. A TATA box is found 29 bases upstream of the transcription initiation site. Two potential binding sites for NF- κ B or NF- κ B-like proteins are found at positions -63 and -77. Iademarco et al., 1992, J. Biol. Chem. 267, 16323-16329. A
10 potential AP-1 binding site is found at position -495 and consensus ets binding sites are present at positions -221, -981 and -1033. GATA binding sequences are found at positions -245 and -259, and three potential octamer binding sites are found at positions -729, -1180 and -1554.

15 Multiple signals induce the expression of VCAM-1 including the cytokines TNF α , IL-1 β and IL-4, bacterial lipopolysaccharide (LPS) and synthetic double-stranded oligonucleotides [poly (I:C)]. It is believed that these are mediated, at least in part, through a 36 base pair enhancer
20 element, located between positions -75 and -35 of the human VCAM-1 gene, which contains the two adjacent KB-like binding sites, termed KL and KR. These elements are believed to bind two distinct transcriptional regulatory factors, both part of the NF- κ B family of transcriptional regulatory factors.

25 A number of VCAM-1 gene regulatory elements, including the GATA, ets, KL and KR binding sites for transcription factors have now been identified. In the following sequence, the KL or "Left" binding site and the KR or "Right" binding site are underlined and labelled.

30

SEQ ID NO. 1

5' - TGGCTCTGCCCTGGGTTCCCTTGAAGGGATTTCCCCTCCGCCTCT-3'

3' - ACCGAGACGGACCCAAAGGGGAACTTCCCTAAAGGGAGGCGGAGA-5'

LEFT (KL) RIGHT (KR)

35

The KL and KR sites differ slightly in their sequences, and gel-shift experiments have shown that they appear to bind different

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transcription factors, both of which are believed to be members of the NF- κ B family of transcriptional regulatory factors. Both sites are necessary, but neither is sufficient alone, for TNF stimulation of VCAM-1 expression. Each of the two binding sites
5 shares characteristics with a different previously described site which binds a member of the NF- κ B family. While not wishing to be bound by theory, it has been proposed that the distinct features of the two sites are responsible for the specificity of the VCAM-1 promotor. Iademarco et al.; *J. Biol. Chem.* 1992,
10 267, 16323-16329.

It can be seen that NF- κ B-like and AP-1-like sites are shared by ICAM-1, VCAM-1 and E-selectin. Oligonucleotides incorporating one or both of these sequences are likely to affect transcription of all three vascular cell adhesion
15 molecules. In contrast, oligonucleotides incorporating a regulatory element unique to a particular gene would be likely to have a specific effect on that gene. For example, GATA and octamer binding sequences are believed to be found in VCAM-1, but not ICAM-1 or E-selectin. Oligonucleotides incorporating one
20 or more of these elements would be expected to inhibit VCAM-1 specifically. As other examples, oligonucleotides comprising the IRE sequence would be likely to differentially inhibit ICAM-1 and oligonucleotides comprising the NF-ELAM-1 sequence are likely to specifically inhibit E-selectin.

It is not necessary for the oligonucleotides to comprise the full length of the transcriptional regulatory factor binding site of the gene in order to be useful in modulating gene transcription. It is only necessary that the oligonucleotide therapeutic be substantially identical to a
25 sufficient portion of the binding region or sequence of the gene as to effectively compete for binding of the transcriptional regulatory factor. Persons of ordinary skill in the art can easily ascertain optimal lengths of oligonucleotide for interaction with particular transcription factors.

30 It has also been found that single strands of nucleic acid can be used in accordance with this invention to modulate gene expression. Thus, a self-complementary unimolecular

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oligonucleotide sequence can "look like" double stranded DNA such that transcriptional regulatory factor can bind to it with concomitant down-regulation of the gene.

In certain embodiments, the two strands of a double-stranded oligonucleotide are covalently crosslinked to each other. Similarly, it may be desirable for a self-complementary oligonucleotide to be covalently crosslinked to stabilize the base-paired regions. Crosslinking of oligonucleotides is disclosed in PCT publication WO 93/18052, the contents of which are herein incorporated by reference.

For therapeutics, an animal suspected of having a disease which can be treated by decreasing the expression of ICAM-1, VCAM-1 and E-selectin is treated by administering oligonucleotides in accordance with this invention. Oligonucleotides may be formulated in a pharmaceutical composition, which may include carriers, thickeners, diluents, buffers, preservatives, surface active agents and the like in addition to the oligonucleotide. Pharmaceutical compositions may also include one or more active ingredients such as antimicrobial agents, antiinflammatory agents, anesthetics, and the like in addition to oligonucleotide.

The pharmaceutical composition may be administered in a number of ways depending on whether local or systemic treatment is desired, and on the area to be treated. Administration may be topically (including ophthalmically, vaginally, rectally, intranasally), orally, by inhalation, or parenterally, for example by intravenous drip, subcutaneous, intraperitoneal or intramuscular injection.

Formulations for topical administration may include ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms or gloves may also be useful.

Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets, or tablets. Thickeners, flavorings,

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diluents, emulsifiers, dispersing aids or binders may be desirable.

Formulations for parenteral administration may include sterile aqueous solutions which may also contain buffers, 5 diluents and other suitable additives.

Dosing is dependent on severity and responsiveness of the condition to be treated, but will normally be one or more doses per day, with course of treatment lasting from several days to several months or until a cure is effected or a 10 diminution of disease state is achieved. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates.

The present invention employs oligonucleotides for use in inhibition of the expression of proteins capable of 15 modulating inflammatory cell adhesion. In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid or deoxyribonucleic acid. This term includes oligomers consisting of naturally occurring bases, sugars and intersugar (backbone) linkages as well as oligomers 20 having non-naturally occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of properties such as, for example, enhanced cellular uptake and increased stability in the presence of nucleases.

25 Specific examples of some preferred oligonucleotides envisioned for this invention may contain phosphorothioates, phosphotriesters, methyl phosphonates, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. Most preferred are those with 30 $\text{CH}_2-\text{NH-O-CH}_2$, $\text{CH}_2-\text{N}(\text{CH}_3)-\text{O-CH}_2$, $\text{CH}_2-\text{O-N}(\text{CH}_3)-\text{CH}_2$, $\text{CH}_2-\text{N}(\text{CH}_3)-\text{N}(\text{CH}_3)-\text{CH}_2$ and $\text{O-N}(\text{CH}_3)-\text{CH}_2-\text{CH}_2$ backbones (where phosphodiester is O-P-O-CH_2). Also preferred are oligonucleotides having morpholino 35 backbone structures. Summerton, J.E. and Weller, D.D., U.S. Patent 5,034,506. In other preferred embodiments, such as the peptide-nucleic acid (PNA) backbone, the phosphodiester backbone of the oligonucleotide may be replaced with a polyamide backbone, the bases being bound directly or indirectly to the

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aza nitrogen atoms of the polyamide backbone. P.E. Nielsen, M. Egholm, R.H. Berg, O. Buchardt, *Science* 1991, 254, 1497. Other preferred oligonucleotides may contain sugar moieties comprising one of the following at the 2' position: OH, SH, SCH₃, F, OCN, 5 O(CH₂)_nNH₂ or O(CH₂)_nCH₃, where n is from 1 to about 10; C₁ to C₁₀ lower alkyl, substituted lower alkyl, alkaryl or aralkyl; Cl, Br; CN; CF₃; OCF₃; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; SOCH₃; SO₂CH₃; NO₂; N₃; NH₂; heterocycloalkyl; 10 heterocycloalkaryl; aminoalkylamino; polyalkylamino; substituted silyl; an RNA cleaving group; a conjugate; a reporter group; an intercalator; a group for improving the pharmacokinetic properties of an oligonucleotide; or a group for improving the pharmacodynamic properties of an oligonucleotide and other substituents having similar properties. Oligonucleotides may 15 also have sugar mimetics such as cyclobutyls in place of the pentofuranosyl group. Modified bases and universal bases, particularly inosine, may also be used in the present invention.

The oligonucleotides in accordance with this invention 20 preferably comprise from about 10 to about 80 nucleic acid base units. It is more preferred that such oligonucleotides comprise from about 12 to 50 nucleic acid base units, and still more preferred to have from about 18 to 35 nucleic acid base units. As will be appreciated, a nucleic acid base unit is a base-sugar 25 combination suitably bound to an adjacent nucleic acid base unit through phosphodiester or other bonds.

The oligonucleotides used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for 30 such synthesis is sold by several vendors including Applied Biosystems. Any other means for such synthesis may also be employed, however the actual synthesis of the oligonucleotides is well within the talents of the routineer. It is also well known to use similar techniques to prepare other 35 oligonucleotides such as the phosphorothioates and alkylated derivatives.

While the present invention has been described with

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specificity in accordance with certain of its preferred embodiments, the following examples serve only to illustrate the invention and are not intended to limit the same.

5 **EXAMPLES**

EXAMPLE 1

Oligonucleotide synthesis: Unmodified DNA oligonucleotides were synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with 10 oxidation by iodine. β -cyanoethyldiisopropyl-phosphoramidites were purchased from Applied Biosystems (Foster City, CA). For phosphorothioate oligonucleotides, the standard oxidation bottle was replaced by a 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the 15 phosphite linkages. The thiation cycle wait step was increased to 68 seconds and was followed by the capping step.

2'-O-methyl phosphorothioate oligonucleotides were synthesized using 2'-O-methyl β -cyanoethyldiisopropyl-phosphoramidites (Chemgenes, Needham MA) and the standard cycle 20 for unmodified oligonucleotides, except the wait step after pulse delivery of tetrazole and base was increased to 360 seconds. The 3'-base used to start the synthesis was a 2'-deoxyribonucleotide.

After cleavage from the controlled pore glass column 25 (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55°C for 18 hours, the oligonucleotides were purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Analytical gel electrophoresis was accomplished in 20% acrylamide, 8 M urea, 45 mM Tris-borate 30 buffer, pH 7.0. Oligodeoxynucleotides and phosphorothioate oligonucleotides were judged from electrophoresis to be greater than 80% full length material.

EXAMPLE 2

35 Evaluation of double-stranded oligonucleotide containing the VCAM-1 KF and KL binding sites on VCAM-1 and ICAM-1 expression: Interference with either the KL binding site or with the KR

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binding site or with both binding sites has been found to interfere with transcription of the gene, leading to diminished production of VCAM-1. The following double-stranded deoxyoligonucleotide, having phosphorothioate internucleotide linkages, was made through solid phase oligonucleotide synthesis of the individual strands:

SEQ ID NO. 2

5' - CTGCCCTGGGTTTCCCTTGAAAGGGATTCCCTCCG -3'
3' - GACGGGACCCAAAGGGAACTTCCCTAAAGGGAGGC -5'

10 KL KR

This oligonucleotide corresponds to the VCAM-1 promoter region (SEQ ID NO. 1) save for truncation of five nucleotides at each of the 5' and 3' ends. The KL and KR sites are underlined; the 15 actual "native" KL and KR sites were used because it is believed that these mimic the natural regulatory elements more closely than consensus sequences.

Equal molar concentrations of the two strands were combined and heated to 65°C for 5 minutes. The strands were 20 allowed to anneal by slowly cooling to 25°C. The resulting double-stranded oligonucleotide was incubated with human microvascular endothelial cells (HMEC) for 15 hours at 37°C. VCAM-1 expression was induced by incubating cells with tumor necrosis factor (TNF- α , 100 u/ml) for 4 hours. Expression of 25 VCAM-1 and ICAM-1, both of which contain binding sites for members of the NF- κ B family of transcription factors, was determined by Northern blot analysis. The results shown in Table 1 demonstrate that double-stranded phosphorothioate VCAM-1 kB binding sites exhibit at least 100-fold selectivity for VCAM-1 compared to ICAM-1, the gene for which also contains a kB 30 binding site. Thus it is believed that the kB binding site(s) on the VCAM-1 gene are distinct from the kB-binding site on the ICAM-1 gene.

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Table 1

Inhibition of VCAM-1 expression by double-stranded phosphorothioate oligonucleotides

5

	<u>ds Oligonucleotide Concentration (nM)</u>	Relative Expression	
		<u>VCAM-1</u>	<u>ICAM-1</u>
10	0	1.0	1.0
	1	0.18	1.32
	10	0.26	1.43
	100	0.19	0.79
	1000	0.12	0.40

15

EXAMPLE 3

Evaluation of double-stranded and single-stranded (self-complementary) oligonucleotides containing the VCAM-1 KF or KL binding site on VCAM-1 and ICAM-1 expression:

The following phosphorothioate oligonucleotides were synthesized corresponding only to the left (KL) or right (KR) transcription factor binding sites of the promotor region of the VCAM-1 gene. The oligonucleotide for the left site comprised:

25 SEQ ID NO. 3

5'- GCCTGGGTTTCCCCTTGAA -3'
3'- CGGACCCAAAGGGAACTT -5'

while the oligonucleotide for the right site comprised:

30 SEQ ID NO. 4

5'- TTGAAGGGATTCCCTCCG -3'
3'- AACTCCCTAAAGGGAGGC -5'

Preliminary data suggest that both SEQ ID NO: 3 and 35 SEQ ID NO: 4 were capable of inhibiting VCAM-1 expression in endothelial cells and smooth muscle cells, showing even higher activity than the longer sequence (SEQ ID NO: 2). Thus, either full length or effective shorter portions of the gene sequence which binds the regulatory factor can be used for the sequence 40 of the therapeutic oligonucleotides of the invention.

An additional oligonucleotide comprising the KL binding site with some additional complexity has the following sequence:

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SEQ ID NO. 12

5' - GCCCTGGGTTCCCTTGAA -3'
3' - CGGGACCAAAGGGAACTT -5'

5 Self-complementary unimolecular oligonucleotide sequences can also be used. The following single stranded DNA oligonucleotides were constructed via solid phase synthesis. They were designed to have a sequence from the KL and KR sites of the VCAM-1 gene:

10 SEQ ID NO. 5 KL

5' - CTGGGTTCCCTTCAGGGAAACCCAG -3'

SEQ ID NO. 6 KR

5' - GAAGGGATTCCTCTTAGGGAAATCCCTTC -3'

15 An additional self-complementary DNA sequence with a phosphorothioate backbone having some less complexity was also synthesized based upon the left site of the binding region of the gene.

SEQ ID NO. 7 KL

20 5' - CTGGGTTCCCTTCAGGGAAACCCAG -3'

SEQ ID NO: 5 was heated to 65° and annealed by slow cooling as described above into its double-stranded (self-complementary form). 100 nM of oligonucleotide was incubated 25 with confluent human primary umbilical cord endothelial cells (HUVEC) in 96 well plates for 18 hours at 37°C. TNF- α (100 u/ml) or LPS (100 ng/ml) was added to the cells and incubated another 18 hours. Cell surface VCAM-1 and ICAM-1 expression was determined by ELISA using VCAM-1 and ICAM-1 antibodies (R & D Systems, Minneapolis MN). The self-complementary phosphorothioate oligonucleotide containing part of the KB binding site for VCAM-1 selectively down-regulates VCAM-1 expression relative to ICAM-1 expression. This is shown in Table 2.

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Table 2

Inhibition of VCAM-1 expression by self-complementary
P=S oligonucleotide (100 nM) containing KL binding site

	Treatment	Absorbance	
		VCAM-1	ICAM-1
10	Control	0.008 ± 0.005	0.180 ± 0.025
	SEQ ID NO:5	0 ± 0	0.109 ± 0.034
	TNF-α	0.18 ± 0.132	0.383 ± 0.112
	TFN-α + SEQ ID NO:5	0 ± 0.034	0.284 ± 0.024
	LPS	0.322 ± 0.052	0.622 ± 0.008
15	LPS + SEQ ID NO:5	0 ± 0.007	0.509 ± 0.068

SEQ ID NO: 5 was also synthesized with a phosphodiester backbone and as a phosphorothioate with methoxy (2'-O-methyl) groups on the 2' position of the nucleotide sugars. These were compared to the phosphorothioate deoxy sequence. The oligonucleotides were heated to 65°C and slowly cooled to allow strands to anneal. Oligonucleotides (100 nM) were incubated with HMEC in 96-well plates for 4 hours in the presence of 7 µg/ml DOTMA/DOPE mixture. TNF-α (100u/ml) was added to the cells and incubated another 18 hours. Cell surface VCAM-1 and ICAM-1 expression was determined by ELISA using VCAM-1 and ICAM-1 antibodies. The results are shown in Table 3.

Table 3

Specific inhibition of VCAM-1 expression by
100 nM SEQ ID NO:5 (P=S)

	Treatment	Relative Expression	
		VCAM-1	ICAM-1
	TNF-α	1.0	1.0
	TNF-α + SEQ ID NO: 5, P=S	0.53	0.97
40	TNF-α + SEQ ID NO: 5, P=S 2'-O-Me	1.19	0.95
	TNF-α + SEQ ID NO: 5, P=O	1.4	0.88
	Negative control DNA	0.94	0.96

45

EXAMPLE 4

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Oligonucleotide interference with VCAM transcription:

Competition for transcription factor binding: A protein was shown to be induced in TNF- α -treated HUVEC cells which selectively bound to the VCAM-1 KL sequence. SEQ ID NO: 5 was 5 shown by gel shift assay to compete with the natural KL sequence on the VCAM-1 gene for binding of this transcription regulatory protein.

CAT assays of VCAM-1 transcriptional activity after oligonucleotide treatment: A CAT reporter plasmid incorporating 10 288 nucleotides upstream of the VCAM-1 transcriptional start site fused in frame to the chloramphenicol acetyltransferase (CAT) gene was transfected into human HMEC cells in the presence or absence of SEQ ID NO: 5 or a mutated version of this 15 oligonucleotide with an altered KL binding region, using standard calcium phosphate transfection techniques. Cells were treated with TNF- α (100u/ml) for approximately 48 hours and cytosolic extracts were prepared. CAT activity was determined by thin layer chromatography and expressed as percent conversion of chloramphenicol to its acetylated form. Results are shown in 20 Table 4:

Table 4

Oligonucleotide effect on VCAM transcription

25	Neg control	0.65
	TNF- α	1.16
	TNF + SEQ ID NO: 5	0.67
	TNF + mut. SEQ ID NO: 5	2.11

30 These results suggest that SEQ ID NO: 5 inhibits the induction of VCAM-1 transcription by TNF- α .

EXAMPLE 5

35 Effect of chimeric multimodal oligonucleotide on VCAM-1, ICAM-1 and E-selectin expression:

An exemplary chimeric oligonucleotide was also prepared having multi-modality. A first region of the chimeric oligonucleotide is double-stranded DNA having a sequence from 40 the left site of the VCAM-1 coding gene which binds transcriptional regulatory factor. A second region of the

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oligonucleotide comprises a DNA having a sequence known to one of the inventors to have significant, traditional antisense activity against VCAM-1. See PCT Patent Application No. PCT/US91/05209 filed July 23, 1991, and U.S. Serial No. 007,997 5 filed January 21, 1993, the entirety of which is incorporated herein by reference. The two regions of the oligonucleotide are joined together by a junction region. The following oligonucleotide (SEQ ID NO: 8) was made; bold type denotes the double stranded phosphorothioate DNA which binds the 10 transcriptional regulatory factor, the underlined portion connotes phosphorothioate DNA having the sequence for antisense interaction with VCAM-1 mRNA and the normal type shows either DNA or RNA which is either a junction region or therapeutically ineffective sequence included for stability.

15

SEQ ID NO. 8

5' - GCCTGGGTTTCCCCCTTG AATTCGA CTGTGTCTCCTGTCTCCGCT -3'

3' - CGGACCCAAAGGGAAAC TTAAACGT GACACAGAGGACAGAGGCGA -5'

20

In this embodiment, the double stranded regulatory factor binding region (bold type) and the antisense strand of the region (underlined) were synthesized to have phosphorothioate backbones. The remaining nucleotides, including the junction region and the sense strand of the antisense region, had wild-type, phosphodiester internucleotide linkages. This multimodal oligonucleotide was evaluated in a preliminary experiment to compare effects of 10 nM oligonucleotides on TNF- α -induced VCAM-1 expression in HUVEC cells.

30

The oligonucleotides were heated to 65°C and slowly cooled to allow strands to anneal. 10 nM of oligonucleotide [multimodal (SEQ ID NO: 8), self-complementary unimolecular (SEQ ID NO: 5) and a mutated version of SEQ ID NO: 5 having a mutated KL site] and 7 μ l/ml lipofectin was added to OPTIMEAM washed confluent, early passaged primary HUVE cells in 96-well plates.

35

After 5 hours, the wells were washed and 20% FCS/DMEM was added for two hours. 100u/ml TNF- α was then added. After an additional 18 hours, cell surface expression of VCAM-1 or ICAM-1 was

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determined by LISA assay on live cells using commercially available antibodies to ICAM-1 and VCAM-1. The results shown in Table 5 are the mean of triplicate assays for each point.

5

Table 5

		<u>VCAM-1</u>	<u>ICAM-1</u>
10	TNF	0.167	0.252
	TNF + SEQ ID NO: 5	0.168	0.236
15	TNF + SEQ ID NO: 8	0.136	0.28
	TNF + mut. SEQ ID NO: 5	0.168	0.24
	Negative control	0.005	0.179

20 This preliminary experiment demonstrated that, at an extremely low oligonucleotide concentration at which the self-complementary oligonucleotide (SEQ ID NO: 5) comprising the VCAM-1 transcription factor KL binding site did not inhibit VCAM-1 expression, the multimodal oligonucleotide containing both the KL site and an antisense region targeted to VCAM-1 (SEQ ID NO: 8) inhibited VCAM-1 expression by approximately 27%. This suggests that the multimodal oligonucleotide may be even more effective than the oligonucleotide comprising only the transcription factor binding site.

30

EXAMPLE 6

Oligonucleotides containing other VCAM-1 regulatory sequences: A double-stranded oligonucleotide containing the GATA sequence element from human VCAM-1 was synthesized as a phosphorothioate.

35 The sequence is as follows:

TTTATCTTCAGTAAAGATAGCCTTTGGAA (SEQ ID NO: 9)
AAATAGAAAGGTCAATTCTATCGAAAACCT

40 A unimolecular self-complementary oligonucleotide containing the same GATA sequence was also synthesized:

CTATCTTCAGTAAAGATAGTTCTATCTTACTCGAAAGATAG (SEQ ID NO: 10)

- 30 -

A double-stranded oligonucleotide containing the proximal ets sequence from human VCAM-1 was also synthesized:

5 GTCGAAGATGAGGAAAGCCTGTATTTTATA (SEQ ID NO: 11)
CAGCTTCTACTCCTTCGGACATAAAAAT

These oligonucleotides were tested under optimized conditions (same as previous example except HUVE cells were 75% confluent) and the results are expressed in Table 6 as percent inhibition of expression after TNF- α induction.

Table 6

15 Effect of regulatory sequence oligonucleotides on
vascular adhesion molecule expression
(Expressed as percent inhibition compared to expression
without oligo, after TNF- α treatment)

		<u>VCAM-1</u>	<u>E-selectin</u>	<u>ICAM-1</u>
20	100 nM KL (SEQ ID NO: 5)	94.26	106.85	37.01
	10 nM KL (SEQ ID NO: 5)	77.05	-23.97	12.20
	1 nM KL (SEQ ID NO: 5)	57.38	5.48	7.48
	100 nM ds GATA (SEQ ID NO: 9)	71.31	23.29	5.51
25	10 nM ds GATA (SEQ ID NO: 9)	94.26	15.75	35.24
	100 nM GATA (SEQ ID NO: 10)	93.44	20.55	-2.56
	10 nM GATA (SEQ ID NO: 10)	81.97	39.04	-9.08
	100nM ets (SEQ ID NO: 11)	81.15	22.60	36.81
30	10 nM ets (SEQ ID NO: 11)	88.52	30.82	13.98

Also in this experiment, SEQ ID NO: 5 was tested at concentrations between 1 nM and 100 nM for ability to inhibit VCAM-1, ICAM-1 and E-selectin expression under optimized conditions. The results are also shown in Table 6. These data indicate that at 100 nM, SEQ ID NO: 5 completely blocks both VCAM-1 and E-selectin induction with only a 37% inhibition of ICAM-1. At lower concentrations, VCAM-1 is still blocked by greater than 50% but there is no effect on either E-selectin or ICAM-1.

At all concentrations of either the bimolecular ets or GATA oligonucleotides or the unimolecular self-complementary 45 GATA oligonucleotide, VCAM-1 expression was inhibited by greater

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than 70% with zero to 40% inhibition of either E-selectin or ICAM-1.

Oligonucleotides containing "native" VCAM-1 enhancer elements are thus able to regulate VCAM-1 expression with a specificity and selectivity which would not be expected with, for example, oligonucleotides containing a consensus regulatory sequence.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Isis Pharmaceuticals, Inc. et al.
5 (ii) TITLE OF INVENTION: Modulation of selected Gene Expression Through Novel Oligonucleotide Interactions

(iii) NUMBER OF SEQUENCES: 12

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: DISKETTE, 3.5 INCH, 1.44 Mb STORAGE

(B) COMPUTER: IBM

20 (C) OPERATING SYSTEM: PC-DOS

(D) SOFTWARE: WORDPERFECT 5.1

(vi) CURRENT APPLICATION DATA:

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(B) FILING DATE: Herewith

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30 (viii) ATTORNEY/AGENT INFORMATION:

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- 33 -

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(C) REFERENCE/DOCKET NUMBER: ISIS-1725

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 46

(B) TYPE: Nucleic Acid

10 (C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TGGCTCTGCC CTGGGTTTCC CCTTGAAGGG ATTTCCCTCC GCCTCT 46

15 (2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36

(B) TYPE: Nucleic Acid

20 (C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CTGCCCTGGG TTTCCCCTTG AAGGGATTTTC CCTCCG

36

(2) INFORMATION FOR SEQ ID NO: 3:

25 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Double

- 34 -

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GCCTGGGTTT CCCCTTGAA

19

5(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Double

10 (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TTGAAGGGAT TTCCCTCCG

19

(2) INFORMATION FOR SEQ ID NO: 5:

15 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

20 (iv) ANTI-SENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CTGGGTTTCC CTTTTCTCAG GGAAACCCAG

30

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

25 (A) LENGTH: 34

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

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(iv) ANTI-SENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GAAGGGATTT CCCTCTTCTT AGGGAAATCC CTTC

34

(2) INFORMATION FOR SEQ ID NO: 7:

5 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

10 (iv) ANTI-SENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CTGGGTTTCC CCTTCAGGGA AACCCAG

27

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

15 (A) LENGTH: 45

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GCCTGGGTTT CCCCTTGAAT TTGCACTGTG TCTCCTGTCT

CCGCT

45

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

25 (A) LENGTH: 31

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

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(iv) ANTI-SENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TTTATCTTTC CAGTAAAGAT AGCCTTTGG A (31)

(2) INFORMATION FOR SEQ ID NO: 10:

5 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 44

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

10 (iv) ANTI-SENSE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CTATCTTCC AGTAAAGATA GTTCTATCTT TACTCGAAAG ATAG (44)

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

15 (A) LENGTH: 31

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE:

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GTCGAAGATG AGGAAAGCCT GTATTTTAT A (31)

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

25 (B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: No

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GCCCTGGGTT TCCCCCTTGAA

19

WHAT IS CLAIMED IS

1. A method for modulating expression of a gene coding for a vascular cell adhesion molecule comprising:
 - 5 selecting a portion of said gene having a binding sequence which binds a transcriptional regulatory factor; and contacting cells containing the gene with an oligonucleotide moiety substantially identical in sequence to at least a portion of said binding sequence of the gene.
- 10 2. The method of claim 1 wherein the vascular cell adhesion molecule is VCAM-1.
- 15 3. The method of claim 2 wherein the transcriptional regulatory factor is selected from the group consisting of: a member of the NF- κ B family, AP-1 and GATA.
- 20 4. The method of claim 2 wherein the DNA sequence which binds a transcriptional regulatory factor is selected from the group consisting of: a TATA box, a kB binding site, KL, KR, an AP-1 binding site, an ets binding site, a GATA binding site, and an octamer binding site.
- 25 5. The method of claim 1 wherein the vascular cell adhesion molecule is ICAM-1.
- 30 6. The method of claim 5 wherein the transcriptional regulatory factor is selected from the group consisting of: a member of the NF- κ B family, AP-1, Sp-1, AP-2, AP-3 and interferon.
- 35 7. The method of claim 5 wherein the DNA sequence which binds a transcriptional regulatory factor is selected from the group consisting of: a TATA box, a CAT sequence, a kB binding site, an AP-1 binding site, an Sp-1 binding site, an AP-2 binding site, an AP-3 binding site, and an interferon response element.

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9. The method of claim 8 wherein the transcriptional regulatory factor is selected from the group consisting of: a member of the NF-kB family, AP-1 and NF-ELAM1.

5 10. The method of claim 8 wherein the DNA sequence which binds a transcriptional regulatory factor is selected from the group consisting of: a TATA box, a kB binding site, an inverted CCAAT sequence, an AP-1 binding site, and an NF-ELAM1 binding site.

10 11. The method of claim 1 wherein said oligonucleotide moiety comprises two complementary strands of DNA.

15 12. The method of claim 11 wherein the two complementary strands of DNA are covalently crosslinked.

13. The method of claim 1 wherein said oligonucleotide moiety comprises a single strand of self-complementary nucleic acid.

20 14. The method of claim 13 wherein the self-complementary nucleic acid strand is covalently crosslinked.

15. The method of claim 1 wherein least one of the linking groups between nucleotide units of the oligonucleotide is a phosphorothioate linking group.

16. The method of claim 1 wherein said modulation is effected through inhibition of transcription of the gene.

30 17. A method for modulating expression of VCAM-1 in cells comprising contacting the cells with a VCAM-1 inhibitory amount of an oligonucleotide moiety having one of the sequences SEQ ID NOS. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12.

35 18. A method for treating atherosclerosis, restenosis or inflammatory disease comprising administering to an animal

- 40 -

having atherosclerosis, restenosis or inflammatory disease a therapeutically effective amount of an oligonucleotide moiety having one of the sequences SEQ ID NOS. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12.

5

19. An oligonucleotide moiety comprising a sequence substantially identical to the sequence of at least a portion of a DNA sequence of a gene which binds a transcriptional regulatory factor, said gene coding for a vascular cellular adhesion molecule.

10

20. The oligonucleotide moiety of claim 19 wherein the vascular cell adhesion molecule is VCAM-1.

15

21. The oligonucleotide moiety of claim 20 wherein the transcriptional regulatory factor is selected from the group consisting of: a member of the NF-kB family, AP-1 and GATA.

20

22. The oligonucleotide moiety of claim 20 wherein the DNA sequence which binds a transcriptional regulatory factor is selected from the group consisting of: a TATA box, a kB binding site, KL, KR, an AP-1 binding site, an ets binding site, a GATA binding site, and an octamer binding site.

25

23. The oligonucleotide moiety of claim 19 wherein the vascular cell adhesion molecule is ICAM-1.

30

24. The oligonucleotide moiety of claim 23 wherein the transcriptional regulatory factor is selected from the group consisting of: a member of the NF-kB family, AP-1, Sp-1, AP-2, AP-3 and interferon.

35

25. The oligonucleotide moiety of claim 23 wherein the DNA sequence which binds a transcriptional regulatory factor is selected from the group consisting of: a TATA box, a CAT sequence, a kB binding site, an AP-1 binding site, an Sp-1

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binding site, an AP-2 binding site, an AP-3 binding site, and an interferon response element.

26. The oligonucleotide moiety of claim 19 wherein the
5 vascular cell adhesion molecule is E-selectin.

27. The oligonucleotide moiety of claim 26 wherein the transcriptional regulatory factor is selected from the group consisting of: a member of the NF-kB family, AP-1 and NF-ELAM1.

10 28. The oligonucleotide moiety of claim 26 wherein the DNA sequence which binds a transcriptional regulatory factor is selected from the group consisting of: a TATA box, a kB binding site, an inverted CCAAT sequence, an AP-1 binding site, and an
15 NF-ELAM1 binding site.

29. The oligonucleotide moiety of claim 19 which comprises two complementary strands of DNA.

20 30. The oligonucleotide moiety of claim 29 wherein the two complementary strands of DNA are covalently crosslinked.

31. The oligonucleotide moiety of claim 19 which comprises a single strand of self-complementary nucleic acid.

25 32. The oligonucleotide moiety of claim 31 wherein the self-complementary nucleic acid strand is covalently crosslinked.

30 33. The oligonucleotide moiety of claim 19 wherein least one of the linking groups between nucleotide units of the oligonucleotide moiety is a phosphorothioate linking group.

35 34. The oligonucleotide moiety of claim 19 selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ

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ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11 and SEQ ID NO: 12.

35. A chimeric oligonucleotide for modulation of
5 expression of a gene comprising a first region having a sequence which is substantially identical to at least a portion of a sequence of said gene, which gene sequence binds a transcriptional regulatory factor, and a second region specifically hybridizable either with a splicing region of pre-
10 mRNA deriving from the gene or with mRNA deriving from the gene.

36. The chimeric oligonucleotide of claim 35 wherein said first and second regions are stabilized against nuclease degradation.
15

37. The chimeric oligonucleotide of claim 35 wherein said first and second regions flank a junction region which is a substrate for a cellular nuclease.
20

38. The chimeric oligonucleotide of claim 37 wherein said cellular nuclease is RNase H or DNase.

39. The chimeric oligonucleotide of claim 36 wherein said first and second regions flank a junction region which is a substrate for a cellular nuclease and the first and second regions are stabilized against nuclease degradation.
25

40. The chimeric oligonucleotide of claim 39 wherein said cellular nuclease is RNase H or DNase.
30

41. The chimeric oligonucleotide of claim 39 wherein said first and second regions are stabilized by phosphorothioate linking groups between nucleotide units of the region.
35

42. A chimeric oligonucleotide for modulation of expression of a gene comprising a first region having a

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sequence which is substantially identical to at least a portion
of a sequence of a gene, which gene sequence binds a
transcriptional regulatory factor, a second region specifically
hybridizable with a splicing region of pre-mRNA deriving from
5 the gene, and a third region specifically hybridizable with
mRNA deriving from the gene.

43. The chimeric oligonucleotide of claim 42 wherein each
of said first, second, and third regions is stabilized against
10 metabolic degradation and flank junction regions which are
labile to metabolic degradation.

44. A method for treating a mammal suspected of having a
disease involving expression of a selected gene, comprising
15 administering to the mammal an oligonucleotide moiety in a
pharmaceutically acceptable carrier or diluent, said
oligonucleotide moiety comprising a nucleotide sequence
substantially identical to the sequence of at least a portion
20 of a sequence of the gene which binds a transcriptional
regulatory factor.

INTERNATIONAL SEARCH REPORT

International application No. PCT/US94/12797

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 48/00; C07H 21/00

US CL : 514/44; 536/23.1, 24.1, 24.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 536/23.1, 24.1, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Sciences, USA, Volume 88, issued September 1991, M.I. Cybulsky et al, "Gene structure, chromosomal location, and basis for alternative mRNA splicing of the human VCAM1 gene", pages 7859-7863, see the entire document.	1-34, 44
Y	The Journal of Biological Chemistry, Volume 267, No. 23, issued 15 August 1992, M.F. Iademarco et al, "Characterization of the Promoter for Vascular Cell Adhesion Molecule-1 (VCAM-1)", pages 16323-16329, see the entire document.	1-34, 44

 Further documents are listed in the continuation of Box C. See patent family annex.

- * Special categories of cited documents:
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document published on or after the international filing date
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "O" document referring to an oral disclosure, use, exhibition or other source
- "P" document published prior to the international filing date but later than the priority date claimed
- "S" document member of the same patent family

Date of the actual completion of the international search

03 MARCH 1995

Date of mailing of the international search report

10 MAR 1995

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12797

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Experimental Medicine, Volume 176, issued December 1992, A.S. Neish et al, "Functional Analysis of the Human Vascular Cell Adhesion Molecule 1 Promoter", pages 1583-1593, see the entire document.	1-34, 44
Y	WO, A, 91/11535 (HOLCENBERG ET AL.) 08 August 1991, see the entire document.	1-44

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/US94/12797**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/US94/12797**B. FIELDS SEARCHED**

Electronic data bases consulted (Name of data base and where practicable terms used):

CA, EMBASE, MEDLINE, DERWENT BIOTECH ABSTRACTS, APS

search terms: vcam, vascular cell adhesion molecule, oligonucleotide, aptamer, transcription factor, gene therapy, DNA binding protein, chimeric, multifunction?, (splice or cap or initiation) site, regulatory element, double, dual, gene expression

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-34 and 44, drawn to methods and oligonucleotides (oligos) for inhibiting expression of vascular cell adhesion molecules.

Group II, claims 35-43, drawn to chimeric oligos having multiple functions.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The two groups are distinct because the chimeric oligos of II are not required to produce the oligos or practice the methods of I. Furthermore, the methods and oligos of I are not required to produce the chimeric oligos of II. Hence the two groups are not linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.